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Improved Production of Plant Isoquinoline Alkaloids by Metabolic Engineering

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Abstract

Higher plants produce diverse low-molecular-weight chemicals such as alkaloids, terpenoids and phenylpropanoid compounds. Among these chemicals, alkaloids are particularly important in medicine due to their high biological activities. However, the low yield of metabolites, especially alkaloids, in plants limits large-scale development of the plant natural product industry. In this chapter, I describe the metabolic engineering of plants to improve the yield and quality of useful secondary metabolites and their potentials. Among alkaloids, I focus on the production of isoquinoline alkaloids (IQAs), since their biosynthesis has been most intensively studied at the molecular level and their application in metabolic engineering has also been intensively examined. Based on our understanding of IQA engineering, I discuss its application to the production of other alkaloids through similar approaches.

Keywords: isoquinoline alkaloid · metabolic engineering · overexpression of rate-limiting enzyme · RNAi · transcription factor

Abbreviations

BBE, berberine bridge enzyme;
Cj, *Coptis japonica*;
CNMT, coclaurine *N*-methyltransferase;
CoOMT, columbamine *O*-methyltransferase;
COR, codeinone reductase;
CYP80A1, berbaminine synthase;
CYP80B1, *N*-methylcoclaurine 3'-hydroxylase;
CYP82G2, corytuberine synthase;
CYP82N2v2 (P6H), protopine 6-hydroxylase;
CYP719A1, canadine synthase;
CYP719A5, chelanthifoline synthase;
CYP719A2/A3, stylophine synthase;
CYP719B1, salutaridine synthase;
IQAs, isoquinoline alkaloids;
MeJA, methyl jasmonate;
NCS, norcoclaurine synthase;
OMT, *O*-methyltransferases;
6OMT, norcoclaurine 6OMT;

4'OMT, 3'-hydroxy-*N*-methyl-coclaurine 4'OMT;

RNAi, RNA interference;

SalAT, salutaridinol-7-*O*-acetyltransferase;

SalR, salutaridine reductase;

SMT, (*S*)-scoulerine 9-*O*-methyltransferase;

THBO, tetrahydroprotoberberine oxidase

1. Introduction

Higher plants produce diverse low-molecular-weight chemicals such as alkaloids, terpenoids and phenylpropanoid compounds (Croteau *et al.*, 2000). Although these compounds are widely used for health and nutrition in humans, alkaloids are particularly important in medicine due to their high biological activities. However, the low yield of metabolites, especially alkaloids, in plants limits large-scale development of the plant natural product industry. Thus, many research groups have tried to establish production systems using the micropropagation of elite plant clones, cell culture and organ culture based on incomplete information regarding biosynthesis of these metabolites. Over the past 20 years, our understanding of the biochemistry and molecular biology of natural products, including alkaloids, has rapidly expanded. Based on knowledge regarding biosynthetic enzymes and their corresponding genes, the modification of productivity and metabolic profiles has become possible (Sato *et al.*, 2007a; Sato & Yamada, 2008). Furthermore, the microbial production of plant metabolites through the reconstruction of plant biosynthetic pathways has become feasible (Chow & Sato, 2013; Hawkins & Smolke, 2008; Minami *et al.*, 2008; Nakagawa *et al.*, 2011; see also Chapter 7). Metabolic engineering in plant cells has advantages and disadvantages. In this chapter, I discuss its potentials and pitfalls based on our experience.

2. Isoquinoline alkaloid biosynthesis and pathway characterization

Due to technological difficulties in the cultivation and relatively slow growth of medicinal plants, plant cell and tissue cultures have been intensively examined for the production of secondary metabolites (Sato & Yamada, 2008; Verpoorte *et al.*, 1991). Whereas the selection of high-yield lines and treatment with an elicitor such as methyl jasmonate has enabled the industrial production of some metabolites, e.g., shikonin-production in selected *Lithospermum erythrorhizon* cells, or paclitaxel production in Pacific yew cell cultures with methyl jasmonate, it is quite difficult to achieve both high productivity and stability for industrial application (Sato & Yamada, 2008). While the development of hairy roots by the transformation of plant cells with *Agrobacterium rhizogenes* provides a solution with unlimited growth, morphological differentiation of roots for physical strength, high potential for the mass-production of valuable secondary metabolites, and greater genetic stability, for fermentation, we need a much faster growth of cells/tissues, higher productivity and improved quality of

metabolites (for details of cell culture techniques, please see Sato & Yamada, 2008).

Recent advances in molecular biology have provided tools for metabolic engineering. The identification of many genes of biosynthetic enzymes and characterization of the spatial and developmental regulation of their expression have clarified their roles in the biosynthesis of secondary metabolites and provided strategies for metabolic engineering such as the trimming of undesired pathways, the enhancement of rate-limiting steps and the introduction of new pathways to produce novel compounds (Sato *et al.*, 2007a,b).

Isoquinoline alkaloid (IQA) biosynthesis provides a good model in metabolic engineering, since it has been intensively studied at the molecular level and experimentally challenged. The biosynthetic pathway for IQAs starts with L-tyrosine, as shown in Figure 1. Among IQAs, the biosynthesis of benzyloisoquinoline alkaloid from norcoclaurine has been the most investigated to date. All of the enzyme genes of the total nine enzymatic reaction steps from norcoclaurine to berberine have been characterized at the DNA level (Fig. 1): a norcoclaurine synthase (NCS; an entry enzyme in IQA biosynthesis, PR10 family such as *Tf*NCS from *Thalictrum flavum* (Lee & Facchini, 2010; Samanani *et al.*, 2004) and a novel dioxygenase-like protein, *Cj*NCS1, from *Coptis japonica* (Minami *et al.*, 2007)), an *N*-methyltransferase (coclaurine *N*-methyltransferase (CNMT, Choi *et al.*, 2002)), three *O*-methyltransferases (OMTs; norcoclaurine 6OMT, 3'-hydroxy-*N*-methyl-coclaurine 4'OMT (6OMT, 4'OMT, Morishige *et al.*, 2000), (*S*)-scoulerine 9-*O*-methyltransferase (SMT, Takeshita *et al.*, 1995), a hydroxylase (CYP80B1, Pauli & Kutchan, 1998), a berberine bridge enzyme (BBE, Dittrich & Kutchan, 1991), a methylenedioxy ring-forming enzyme (canadine synthase; CYP719A1, Ikezawa *et al.*, 2003), and a tetrahydroprotoberberine oxidase (THBO, Gesell *et al.*, 2011; Matsushima *et al.*, 2012).

>>Insert Figure 1 here

The number of available enzyme genes is growing rapidly (Figs. 1 and 2): the genes of corytuberine synthase in aporphine biosynthesis (CYP82G2, Ikezawa *et al.*, 2008); those of salutaridine synthase (CYP719B1, Gesell *et al.*, 2009), salutaridine reductase (SalR, Ziegler *et al.*, 2006), salutaridinol-7-*O*-acetyltransferase (SalAT, Grothe *et al.*, 2001), thebaine 6-*O*-demethylase and codeine *O*-demethylase (T6ODM, CODM, Hagel & Facchini, 2010) and NADPH-dependent codeinone reductase (COR, Unterlinner *et al.*, 1999) in morphinan alkaloid biosynthesis; those of chelanthifoline synthase (CYP719A5, Ikezawa *et al.*, 2009), stylopine synthase (CYP719A2/A3,

Ikezawa *et al.*, 2007), (*S*)-cis-*N*-methylstylopine 14-hydroxylase (Beaudoin & Facchini, 2013), protopine 6-hydroxylase (CYP82N2v2, Takemura *et al.*, 2013), sanguinarine reductase (Vogel *et al.*, 2010) and dihydrobenzophenanthridine oxidase (DBOX, Hagel *et al.*, 2012) in benzophenanthridine biosynthesis; those of berbamin synthase (CYP80A1, Kraus & Kutchan, 1995) in the bis-benzylisoquinoline alkaloid pathway; those of *O*-methyltransferases (Dang & Facchini, 2012) and a 10-gene cluster (three *O*-methyltransferases (PSMT1-3), four cytochrome P450s (CYP82X1/2, CYP82Y1 & CYP719A21), an acetyltransferase (PSAT1), a carboxylesterase (PSCXE1) and a short-chain dehydrogenase/reductase (PSSDR1)(Winzer *et al.*, 2012) in noscapine biosynthesis and those of *O*-methyltransferases in emetine biosynthesis (IpeOMT1/2/3, CiOMT, Cheong *et al.*, 2011; Nomura & Kutchan, 2010).

>>Insert Figure 2 here

While many homologues are annotated after cloning of the first enzyme gene, we should pay close attention to the properties of each enzyme, since pathways in secondary metabolism are often diversified and connected in a complicated manner, based on the different substrate and reaction specificity of the enzyme acquired during the evolution of each species. For example, while *Coptis japonica* columbamine *O*-methyltransferase (CjCoOMT) can use both tetrahydrocolumbamine and columbamine in palmatine biosynthesis, columbamine was once thought to be the exclusive substrate of CoOMT in *Berberis* plants (Morishige *et al.*, 2002). Similarly, CNMT of *Coptis* and *Berberis* showed different substrate specificities; *i.e.*, the *Coptis* enzyme could *N*-methylate norlaudanoline, while the *Berberis* enzyme could not. In this sense, the knowledge needed for both metabolic engineering and synthetic biology is still incomplete, and such information is greatly needed. We should be careful in the annotation of an enzyme gene simply based on homology, since only a few changes in amino acids can modify reaction specificities; *e.g.*, Ipecac OMTs in emetine biosynthesis (Fig. 3; Cheong *et al.*, 2011; Nomura & Kutchan, 2010).

>>>Insert Figure 3 here

3. Metabolic pathway engineering

3.1. Pathway engineering with overexpression of the rate-limiting enzyme and transcription factors in alkaloid biosynthesis

With an increase in the molecular tools available in metabolic engineering, we can now modulate the metabolic flow of plant cells. Among several strategies, overexpression of the gene for the enzyme in the rate-limiting step is commonly examined to improve the yield (Fig. 4). The overexpression of *C. japonica* SMT in the original cells was the first successful application of genetic engineering to IQA biosynthesis to increase berberine production (Sato *et al.*, 2001), while 4'OMT overexpression was later successfully applied in intact plants (Inui *et al.*, 2012). Allen *et al.* (2008) also reported that transgenic opium poppy with overexpression of *SalAT* had more than 30% greater total alkaloids than the control in three independent trials over 3 years.

However, overexpression of an endogenous gene can (often) induce the co-suppression of the gene and decrease the production of the end-metabolites (Jorgensen, 1995; Takemura *et al.*, 2010a). Thus, heterologous expression of a homologue isolated from a different plant species is recommended. For example, *Cj6OMT* was expressed in California poppy (*Eschscholzia californica*) cells to overcome the rate-limiting step (Inui *et al.*, 2007). Note that bottleneck step(s) may differ among plant species and cell lines due to variations in gene expression. In fact, the overexpression of *Cj4'OMT* was less effective for increasing alkaloid production than that of 6OMT in *E. californica* cells, whereas 4'OMT was effective in *C. japonica* plants (Inui *et al.*, 2007, 2012). Changes in the bottleneck step may also occur after modification of the enzyme expression level through metabolic engineering.

Since there are multiple bottleneck steps and these limiting steps can change with overexpression of the enzyme in the limiting step, overall regulation of the expression levels of biosynthetic genes in the pathway is the preferred strategy. Whereas information about the transcription factors in alkaloid biosynthesis is very limited, several transcription factors, such as the ERF, WRKY and bHLH families in alkaloid biosynthesis, including IQA biosynthesis, have been isolated and characterized (Kato *et al.*, 2007; Yamada *et al.*, 2011a,b; Yamada & Sato, 2013). While experimental results suggest that the activities of native transcription factors can be fine-tuned spatially and temporally (see Yamada & Sato, 2013), the successful application of the overexpression of heterologous *AtWRKY* transcription factor in opium poppy to increase the morphinan alkaloid content (Apuya *et al.*, 2008) suggests that transcription factors may be used to improve the yields of IQAs and other alkaloids.

>>Insert Figure 4 here

3.2. Pathway engineering with trimming of pathways and the introduction of new branch pathways

While an increase in the metabolite yield is important for industrial application, modification of the metabolite profile by the introduction of a new metabolic branching point and/or the trimming of undesired pathways is another important approach in drugs development.

To increase metabolite diversity, we ectopically expressed *CjSMT*, which is involved in berberine biosynthesis but not in benzophenanthridine alkaloid biosynthesis, in California poppy (*E. californica*) cells (Fig. 5). *CjSMT* expression not only produced columbamine (oxidized product of tetrahydrocolumbamine) in transgenic *E. californica* cells (Sato *et al.*, 2001) but also induced the accumulation of novel products, *i.e.*, allocryptopine and 10-hydroxychelerythrine, derived from *CjSMT* reaction products by the endogenous enzyme reactions in *E. californica* cells, indicating that the alkaloid profile in transgenic cells was further diversified by the introduction of a branched pathway (Takemura *et al.*, 2010b).

>> Insert Figure 5 here

While an increase in metabolite diversity is preferable for the screening of novel products, the reduction of undesired metabolic diversity is also required to prepare a single purified chemical and increase the metabolic flow to the desired compound. Among the methods for gene silencing (*e.g.*, antisense, RNA silencing, knockout with T-DNA insertion, mutation,.. etc.), RNA silencing with double-stranded RNA molecules (RNA interference; RNAi with short interfering (si) RNA or micro (mi) RNA, co-suppression of gene expression with overexpression of a target gene, or virus-induced gene silencing (VIGS)) is the most powerful and reliable, although Transcription Activator-Like Effector Nucleases (TALEN) could be useful for preparing knockout mutant and novel mutants through homologous recombinants (Borgio, 2009; Desgagné-Penix & Facchini, 2012; Sato, 2005; Winzer *et al.*, 2012; Zhang *et al.*, 2013).

Successful RNAi of BBE in California poppy cells induced the accumulation of reticuline, a substrate of BBE (Fujii *et al.*, 2007), whereas antisense RNA of BBE produced little accumulation of reticuline in transgenic cells (Park *et al.*, 2002) (Fig. 6). However, there is another pitfall in effective gene silencing; *e.g.*, an enhanced branch pathway from the accumulated reticuline to produce a byproduct, *i.e.*, 7-*O*-methylreticuline, which could scarcely be detected in control cells. In addition,

suppression of a certain enzyme in a metabolic pathway may induce the unexpected disturbance of the pathway; *e.g.*, RNAi of COR genes in opium poppy induced the accumulation of a far-upstream precursor, (*S*)-reticuline (Allen *et al.*, 2004). The disturbance of metabolism by the down-regulation of enzyme expression can be partly explained by the accumulation of an intermediate and feedback regulation of the pathway, or the disruption of an enzyme complex (metabolome) to catalyze sequential reactions, or off-target effects of RNAi on other unexpected gene targets (Allen *et al.*, 2004, 2008).

>>> Insert Figure 6 here

While simple overexpression of the rate-limiting step enzyme/transcription factors, introduction of a branch pathway and trimming of a pathway through the use of gene silencing are currently applied in metabolic engineering in alkaloid biosynthesis, the combination of these strategies provides a more effective approach for improving both the quantity and quality of metabolites. For example, trimming of the endogenous pathway with the introduction of new branch was critical for drastic modification of the metabolite in blue rose production; *i.e.*, the accumulation of sufficient blue pigment, delphinidin, was only achieved by the combined expression of the gene for a novel blue pigment biosynthetic enzyme, *i.e.*, flavonoid 3',5'-hydroxylase, and the down-regulation of endogenous dihydroflavonol 4-reductase by RNAi to shut down the undesired red pigment pathway (Katsumoto *et al.*, 2007). Such a combined approach can be performed with a transcription factor and an enzyme for a rate-limiting step that is not under the control of the introduced transcription factor. This can provide greater flexibility in metabolic engineering.

4. Perspectives

While the biosynthetic pathways for many metabolites still require considerable characterization including of the involved enzymes and their genes, continued advances in molecular and genomic techniques will surely expand the production of useful natural products, especially alkaloids. Particularly, whole genome sequence data bring considerable information on biosynthesis along with rapidly accumulating RNA sequence data. The combined analysis of transcriptomes, proteomes, metabolomes and genomes provides a new approach for identifying the candidate genes in uncharacterized pathways (Dang & Facchini, 2012; Desgagné-Penix *et al.*, 2010, 2012; Farrow *et al.*, 2012; Takemura *et al.*, 2010b; Winzer *et al.*, 2012). The

establishment of transcriptome and proteome databases for the investigation of natural product metabolism in non-model plant systems has provided useful information for metabolic engineering. Desgagné-Penix *et al.* (2010) showed that profiling of the more-abundant proteins in elicitor-treated opium poppy cell cultures revealed several uncharacterized enzymes that potentially catalyze steps in sanguinarine biosynthesis.

Structural biology also provides important cues for isolating key biosynthetic enzymes in pathways and their molecular functions, although structural information regarding the biosynthetic enzymes in secondary metabolism is very limited (Ilari *et al.*, 2009; Wallner *et al.*, 2012; Winkler *et al.*, 2009).

Whereas we can use both plant and microbial cells as a platform for metabolite production (Chow & Sato, 2013; see also Chapter 7), we should be aware of their pros and cons. For instance, while plant cell or tissue cultures can produce more divergent chemicals than microbial systems, their complexity and instability are problematic for industrial production. Microbial systems enable alkaloid production on a relatively large scale within a short period, but require more molecular informations on the biosynthetic pathways and enzymes, whereas the emergence of synthetic biology offers the possibility of improving the production of useful products through the design and engineering of complex biological systems. While computer-assisted molecular design software is being developed to facilitate the synthesis of novel compounds with drug-like properties and desired activities, the use of information science for the reconstruction of metabolic pathways, *i.e.*, “retrobiosynthetic” systems, will require more experimental data on the identification and analysis of biosynthetic enzymes and pathways. However, technological progress has been quite rapid and provides us with many options. The potentials of metabolic engineering in secondary metabolism are unlimited.

5. Conclusion

Metabolic engineering as well as synthetic biological approaches are at the forefront in natural product research. Our experiences in IQA biosynthesis provide new clues for the molecular characterization of biosynthetic pathways and novel production systems for natural products. Technological advances, including next-generation sequencing, offer a bright future for the production of not only IQAs but also other alkaloids. This high potential in natural product research will require more sophisticated techniques as we move from metabolites to drug discovery.

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Figure 1. Main pathway in isoquinoline alkaloid biosynthesis. Major isoquinoline alkaloids, such as aporphine-, benzophenanthridine- and protoberberine-type isoquinoline alkaloids, are synthesized through a common pathway derived from norcoclaurine to reticuline. Aporphine alkaloids are synthesized from (*S*)-reticuline by the reaction of corytuberine synthase (CYP80G2). Protoberberine and benzophenanthridine alkaloids are synthesized from (*S*)-scoulerine produced from (*S*)-reticuline by the reaction of berberine bridge enzyme (BBE). Ipecac and some other IQAs are synthesized from dopamine without norcoclaurine biosynthesis. The names of the biosynthetic enzymes for which cDNA has been isolated and characterized are shown. Abbreviations for biosynthetic enzymes are defined in the text, except for TYDC (tyrosine/DOPA decarboxylase; Facchini *et al.*, 2000) and TyrAT (tyrosine aminotransferase; Lee & Facchini, 2011).

Figure 2.

Extended pathways in isoquinoline alkaloid biosynthesis; from reticuline to protoberberine, benzophenanthridine, morphinan and noscapine alkaloids (a) Protoberberine and benzophenanthridine alkaloid biosynthesis has mainly been characterized in *Coptis japonica* (*Cj*) and *Eschscholzia californica* (*Es*) cells, while some genes have been isolated from *Papaver somniferum* (*Ps*). (b) Morphinan and noscapine biosynthesis were characterized in *Papaver somniferum*.

The names of the biosynthetic enzymes for which cDNA has been isolated and characterized are shown. Abbreviations for biosynthetic enzymes are defined in the text, except for CoOMT (columbamine *O*-methyltransferase), and 7OMT (reticuline 7-*O*-methyltransferase).

Figure 3.

Ipecac OMT alignment and reaction specificity

Figure 4.

Yield improvement by the ectopic expression of *C. japonica* (*Cj*) 6OMT in California poppy cells.

Figure 5.

Metabolic diversification by the introduction of a branch pathway

The introduction of a novel branch pathway in California poppy induced the production of uncommon protoberberine-type alkaloids, whereas major products were further

metabolized to di-methoxy-type benzophenanthridine alkaloids by endogenous enzymes.

Figure 6.

Quality modification by the trimming of a pathway

Knockdown of BBE expression with double-stranded RNA strongly reduced endogenous alkaloid accumulation and increased the accumulation of a precursor, reticuline, and some metabolites such as 7-*O*-methylreticuline.

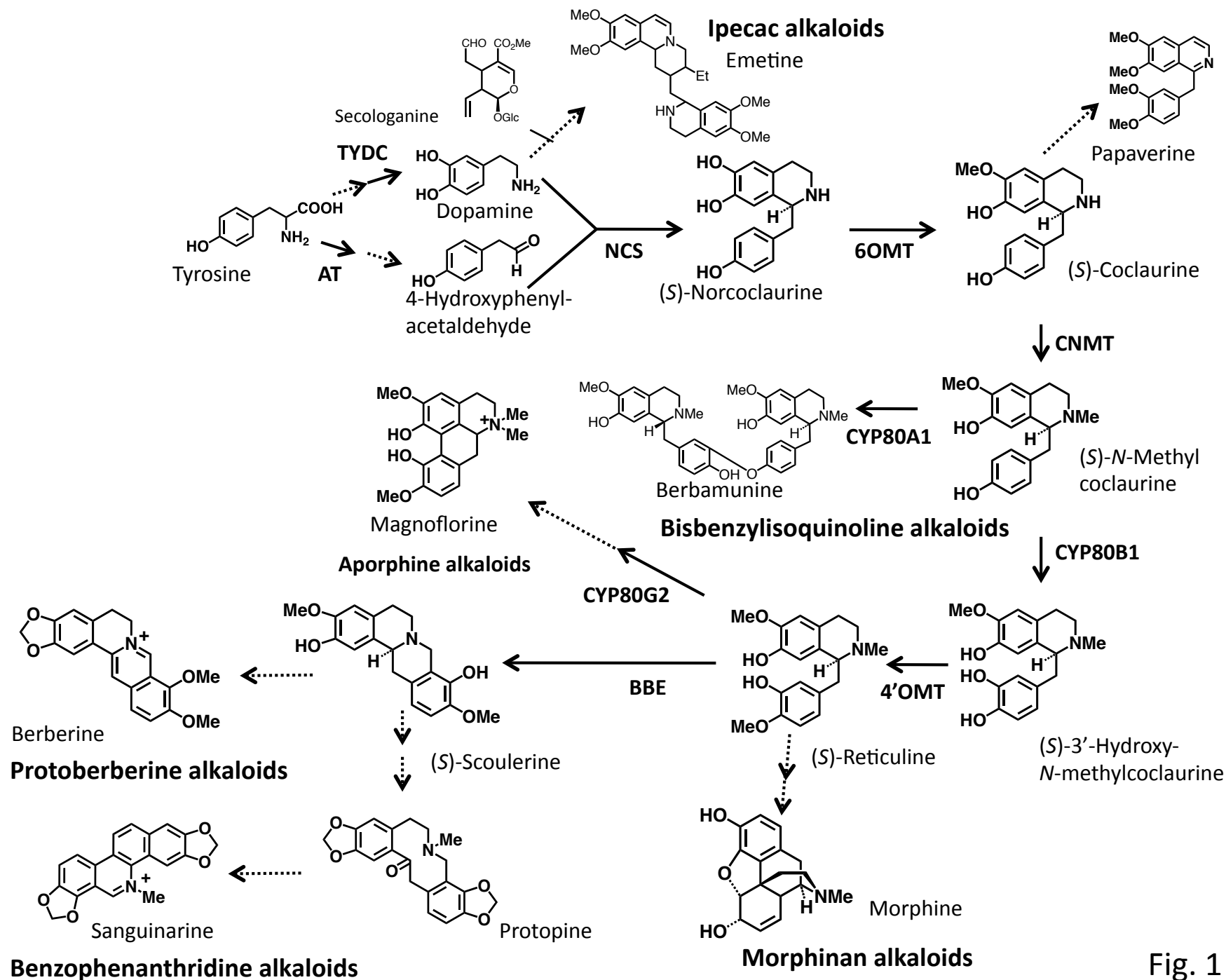
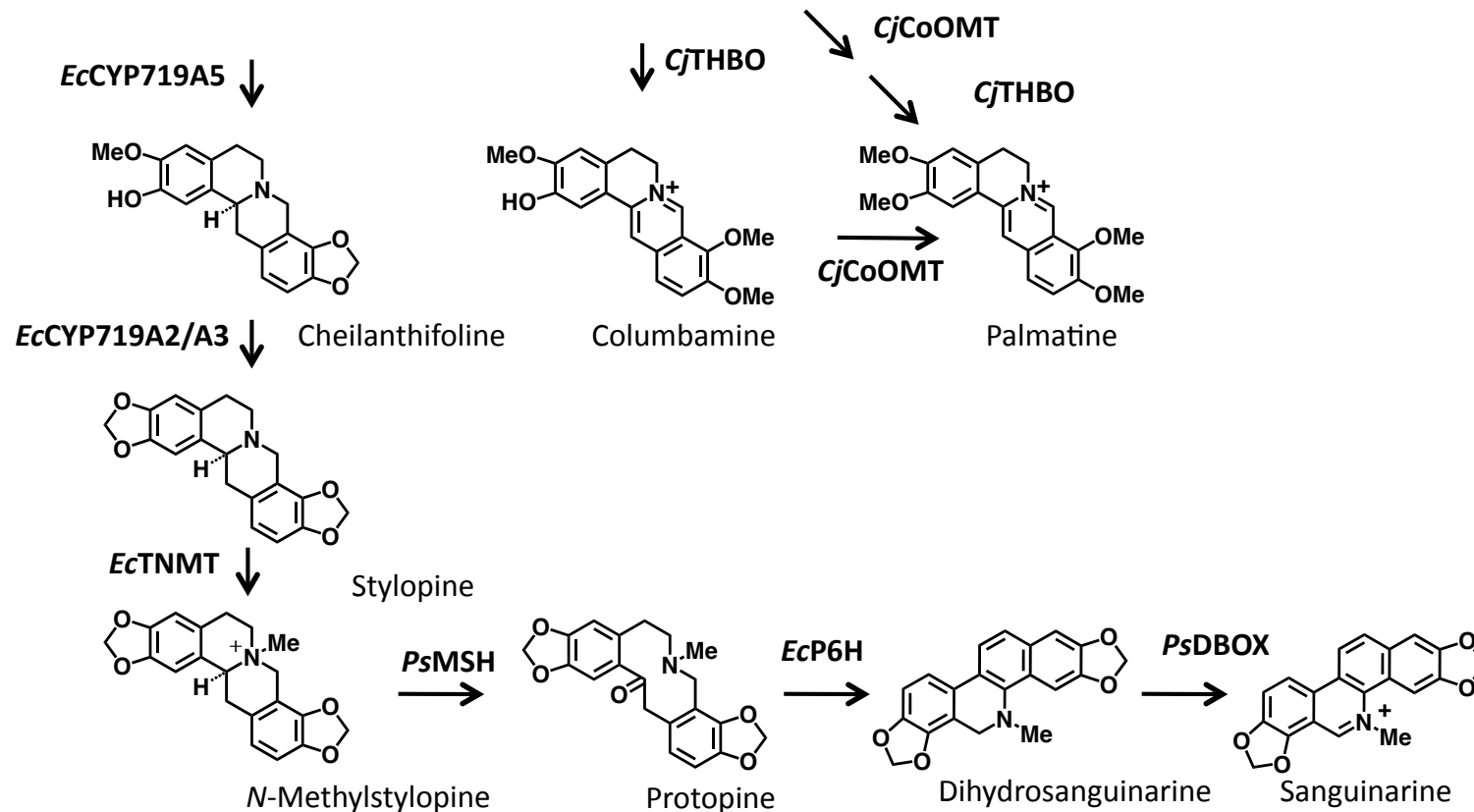
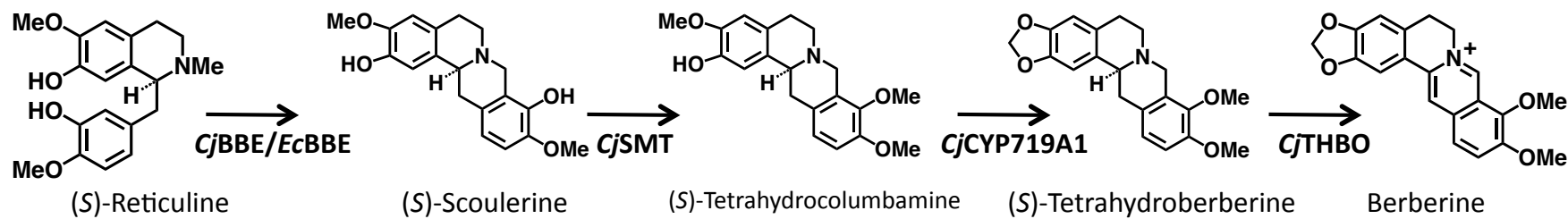


Fig. 1

Protoberberine pathway



Benzophenanthridine pathway

Fig. 2 (A)

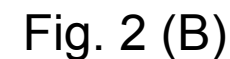


Fig. 2 (B)

	1C	2C	3C	4C	5C	6C	7C	8C	9C	10C					
IpeOMT1	MEIVSSSSAELLRAQTE	STGV S QNEPALKCALOI	IPDAIKQH	KFMNLSELTSALPIPSKAPCIHRIMRLIVAA	AOENEC	ALTSI	RLIL								
IpeOMT2	MEIVSSSSAELLRAQTE	STOL S QNYASLKCALHL	IPDAIKQH	KFMNLSELTSALPIPSKAPCIHRIMFMIVAA	AOENEC	DLTPI	LLLL								
IpeOMT3	MEIVSSSSAELLRAQTE	STOL G HYTPALKCAVHL	IPDAIKQH	KFMNLSELTSALSIPIPSKAPCIHRIMRIIVIA	AOENEC	SLTSA	RHEL								
CiOMT	MEIVSSSSAELLRAQTE	STOL S QNYASLKCALHL	IPDAIKQH	KFMNLSELTSALPIPSKAPCIHRIMFMIVAA	AOENEC	DLTPI	LLLL								
	11C	12C	13C	14C	15C	16C	17C	18C	19C	20C					
IpeOMT1	KDDPLHIRAIVLSEEHIAIVFWALSEL	FNDDVAP	ATAE	KS	WD	TSQDPELRV	DEANAGDSLI	AKIVTE	R	L	E	LRSIVCV	E	TIA	
IpeOMT2	KDDPINIRAIIVLLEHFAALLFWALSEL	CNDDATP	VTAE	KS	WD	TSQDPEERL	DEANAGDS	ELISKIVTE	R	V	E	LRSIVCV	E	TIA	
IpeOMT3	KDDPINIRAIIVLLEHFAIVFWALSEL	CNDDVSP	ATAE	KS	WD	VTSDPELRV	DEANAGDS	LI	AKIVTE	R	V	E	LRSIVCV	S	TIA
CiOMT	KDDPINIRAIIVLLEHFAALLFWALSEL	CNDDATP	VTAE	KS	WD	TSQDPEERL	DEANAGDS	ELISKIVTE	R	V	E	LRSIVCV	E	TIA	
	21C	22C	23C	24C	25C	26C	27C	28C	29C	30C					
IpeOMT1	RSIAFA	TELKCTVODLPEAVANEC	D	NLD	VA	EM	DFVPSADAILK	SILBWSDEN	CVKILK	CKRAISGKDE	FVIDIDVVELQKK--	AGDD			
IpeOMT2	RSIAFA	TELKCTVODLPEAVANEC	D	NLD	VA	EM	DFVPSADAILK	SILBWSDEN	CVKILK	CKRSIPGKDE	FVIIIEVVELEKN--	AGNEY			
IpeOMT3	FAIAFA	TELKCTVODLPEAVANEH	D	NLD	VA	EM	EFVPSADSILK	SILBWSDEN	CVKILK	CKRAIP--	KN	FVIIVDELLK	NRGHGAEEH		
CiOMT	RSIAFA	TELKCTVODLPEAVANEC	D	NLD	VA	EM	DFVPSADAILK	SILBWSDEN	CVKILK	CKRSIPVKDE	FVIIIEVVELEKN--	AGNEY			
	31C	32C	33C	34C	35C	36C									
IpeOMT1	PGLDN----	MLMCMIVLFSKERKKEFAKL	SLA	SD	G--IVRTFGWNCIMELSP--										
IpeOMT2	AGLEN----	LMEMFVLFSEKERRKEFAKL	SLA	SD	K--FIPALDSWCIIELSP--										
IpeOMT3	DEATESPLSCMEMIVLFSKERKKEFAKL	SLA	ND	KYNITPVLGSTSLIEVNFYN											
CiOMT	AGLEN----	LMEMIVLFSKERKKEFAKL	SLA	SD	K--FIPALDSWCIIELTP--										

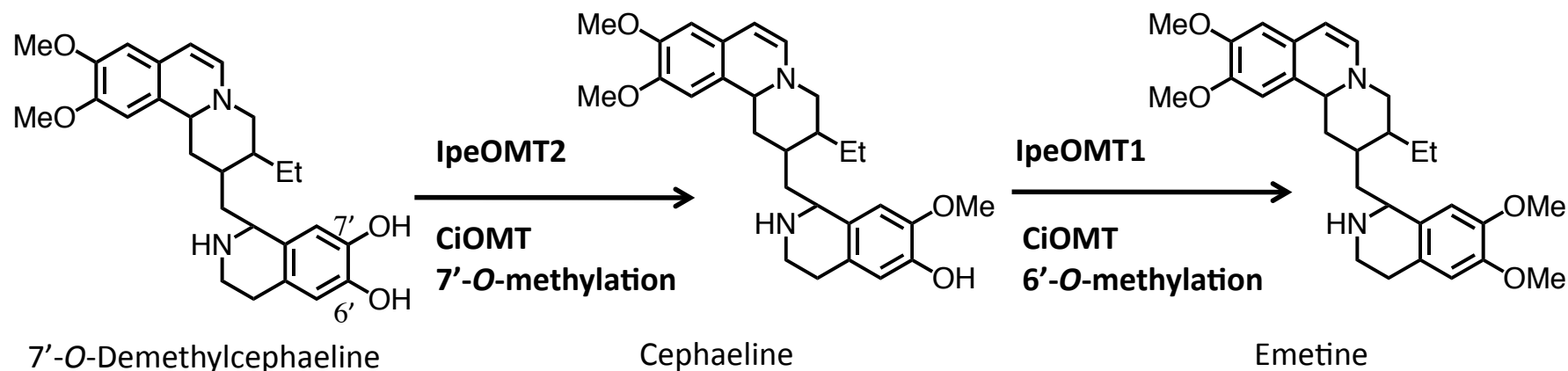


Fig. 3

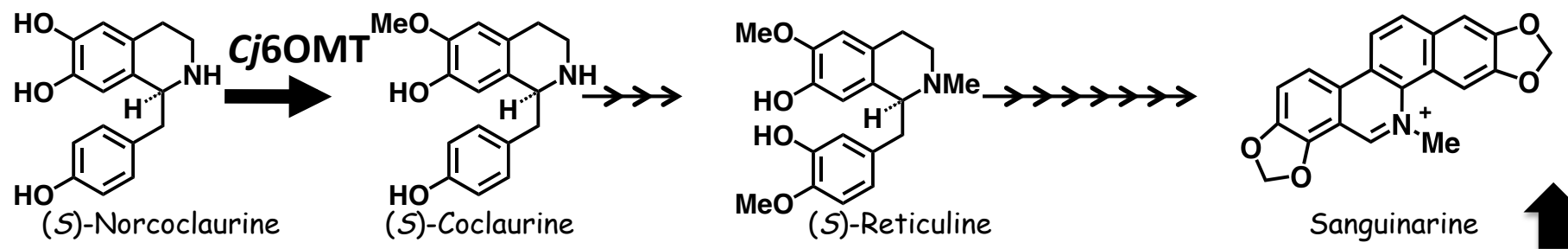


Figure 4

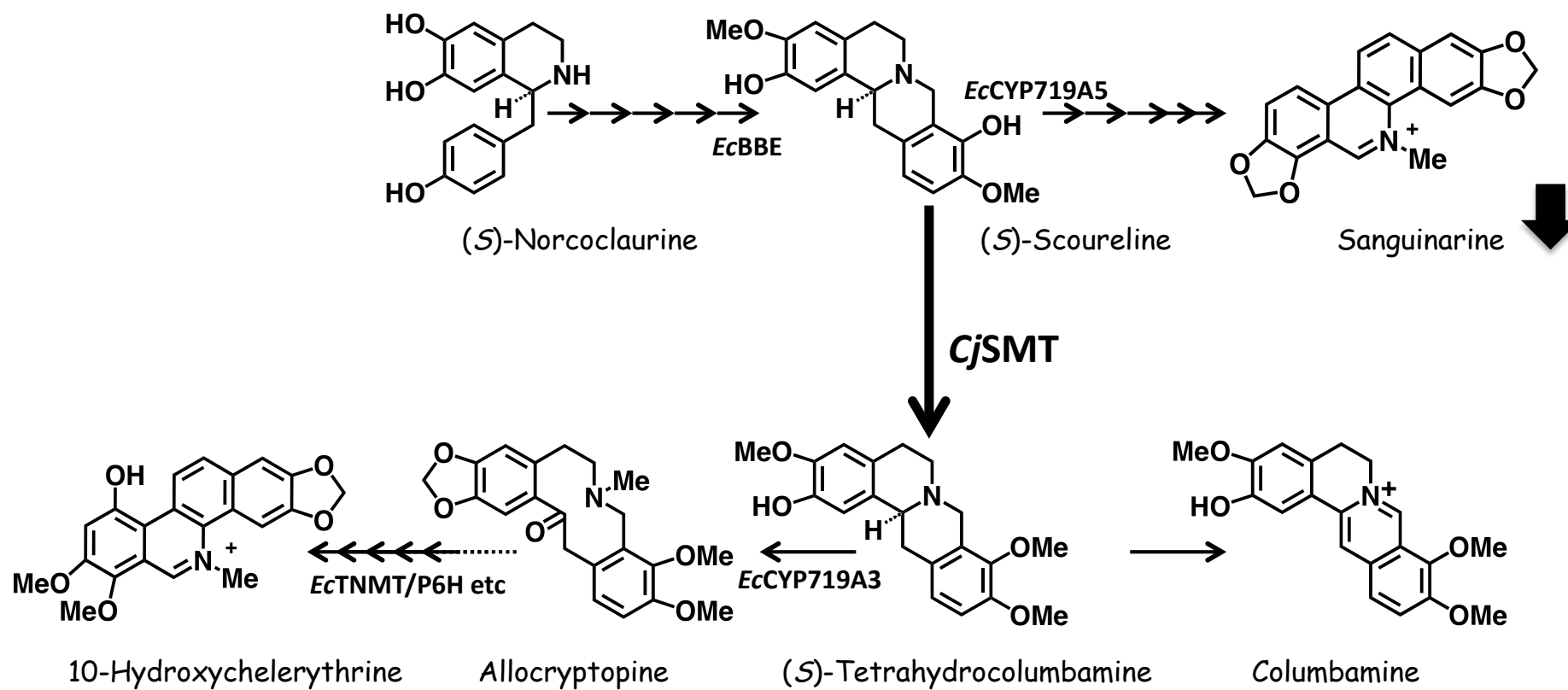


Figure 5

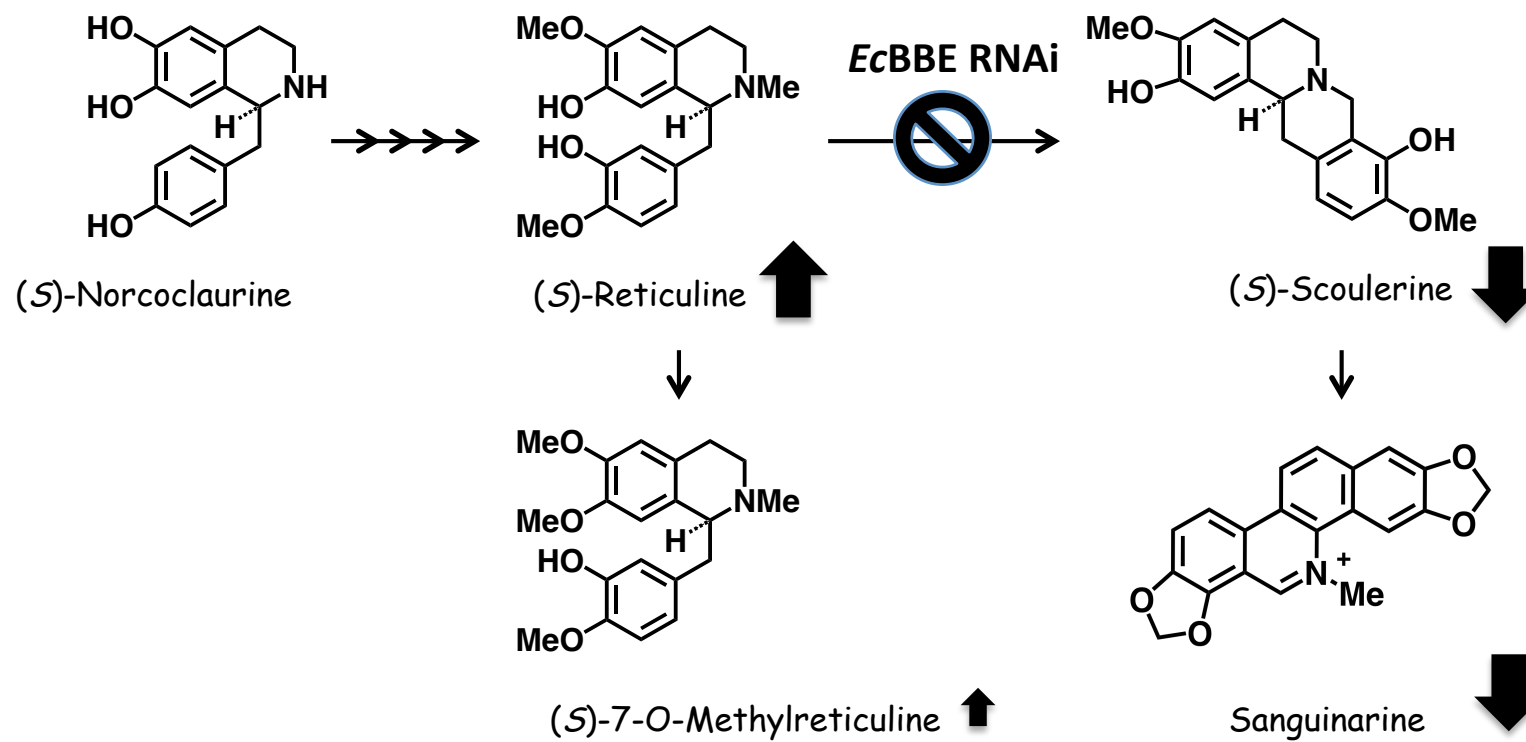


Figure 6